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Specificity of the Effects of Lead on Brain Energy Metabolism for Substrates Donating a Cytoplasmic Reducing Equivalent

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Lead chloride, at concentrations of 67 μM in the incubation media, inhibits the potassium-stimulated respiration (3 to 30mM increase in K^+) of rat cerebral cortex slices. The inhibition observed was dependent upon the substrate provided. In the presence of 10mM glucose or lactate, 35–50% inhibition of the response was observed, but no evidence of an effect could be observed when 10mM pyruvate served as substrate. In consonance with these observations, spectral measurements during the course of the response revealed an attenuation of the initial NAD(P)H oxidation followed by a substantial accumulation of the intermediate in slices metabolizing glucose, but not those metabolizing pyruvate. *In vivo* treatment of adult rats with six intraperitoneal injections spaced over a 14-day period gave rise to essentially similar findings at cerebral cortical lead concentrations averaging 0.41 μg/g (range = 0.34–0.52) and above. No effect was observed at brain lead concentrations averaging 0.17 μg/g.

These data suggested that lead interfered with the oxidation of the NAD(P)H produced by the initial oxidations of glucose in the cytoplasm. Inhibition of cytoplasmic NAD(P)H oxidation by brain mitochondria is of peculiar importance in a tissue relying almost exclusively upon the metabolism of glucose *in vivo*.

Introduction

The picture developed in animal models of lead encephalopathy involves a primary lesion in cerebellar capillaries which is followed by degenerative changes in astrocytic foot processes and then neuronal degeneration (*1*). This sequence of events is taken to indicate that alterations of the blood–brain barrier to lead is the initial insult in the development of lesions in young animals. However, as Michaelson and Sauerhoff (*2*) have pointed out, the production of these changes requires very high doses of lead and results in gross abnormalities of nervous system function. This gross pathology probably obscures the development of more subtle functional changes such as the recently documented behavioral changes which have an intriguing resemblance to hyperkinesis in children both symptomatically and phar-

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macologically (*2, 3*). These latter changes are observed without evidence of gross histopathology.

In the same light, it would seem that the rarity of lead encephalopathy in lead-exposed adults (either animal or man) does not state with any certainty the lack of lead effect on higher functions of the nervous system. These effects would be very difficult to assess in individual patients because of the inability to generate a proper control or normal value. Consequently, we feel that the so-called insenitivity of the adult brain to the effects of lead needs re-examination. This is particularly important when the much longer period of lead exposure as an adult is taken into account and the fact that lead encephalopathy in the child really involves acute or at least subacute exposures to very high doses of lead.

In order to immediately address the question as to the inherent sensitivity of adult rat brain to lead, we have utilized a spaced intraperitoneal (IP) injection schedule. We judged the relevance of our data on the basis of the lead concentration achieved in brain at the end of the treatment period. This is basically to avoid the problem of the very low absorption rate of lead from the gastrointestional tract. Secondly, we have chosen a general model of CNS function which we feel avoids the interpretive problems of extrapolating behavioral measurements in animals to man. While the model has the disadvantage of not producing data directly translatable into functionalities at the whole animal level, it does unquestionably address the properties of brain which are basic to these functions.

Methods

Male Sprague-Dawley rats (200–400 g) were exposed to $PbCl_2$ dissolved in distilled water by the intraperitoneal route over a 2-week period. The total doses administered for the period were 3, 12, and 60 mg Pb/kg body weight in six evenly divided doses. The volume of the injection was 2.0 ml/kg. Controls were injected at the same frequency with an equivalent volume of distilled water. Animals were sacrificed 2 days following the final injection. Slices were prepared from the outer layer of the cerebral cortex and incubated as previously described (4).

The incubation media consisted of NaCl, 127*mM*; $MgCl_2$, 1.3*mM*; NaH_2PO_4, 1.3*mM*; KCl, 3.0*mM*; $CaCl_2$, 0.75*mM*, plus glucose, lactate or pyruvate at a concentration of 10*mM* and either $NaHCO_3$, 26*mM*, or glycylglycine, 30*mM*, as buffer. The media was continually saturated with 95% O_2/5% CO_2 when bicarbonate served as buffer and 100% O_2 when glycylglycine was used. The pH of the media was 7.4. Tissues were incubated under the superfusion conditions routinely employed in our laboratory (*4*).

After a 30-min preincubation period, the media potassium concentration was increased from 3 to 30*mM*. Spectral, oxygen uptake and lactic acid measurements were made as previously described (*4, 5*).

In vitro experiments were conducted similarly. Lead was introduced, as the chloride, 20 min prior to the addition of potassium.

Lead determinations were made in a separate group of animals exposed to the same IP injection schedule described above. Flameless atomic absorption spectrophotometry employing a graphite furnace was used for the analysis following solubilization of the tissue with 1.0 ml 24% tetramethylammonium hydroxide in methanol. Some difficulties were encountered in obtaining consistent recoveries from lead-treated animals so the method of standard additions was employed. Full details of the method will be published elsewhere (Ulmer, in preparation).

Results

As a means of estimating the gross toxicity of the IP injection schedule used to expose animals to lead, body weight of the animals were periodically measured. These results are shown in Figure 1. Only at the highest dose used, 60 mg/kg total for the 14-day period, was there any marked effect on body weight gain of young adult rats. Even in this case, the effect appeared to be temporary. After a 4–5 day lag, animals on this dose began to gain weight at a rate parallel to control animals, despite the continued administration of lead.

Cerebral cortex slices taken from lead-exposed animals were observed to show marked changes in their metabolic responses to an increase in media potassium concentration from 3 to 30*mM*. This was particularly evident in spectral measurements of tissue NAD(P)H* (Fig. 2) when 10*mM* glucose served as substrate. Lead was seen to shorten the normal oxidative phase of the NAD(P)H response and markedly to enhance both the rate and extent of NAD(P) reduction in the second phase of the response. The rate at which this latter effect

*The term NAD(P)H is used to refer to both NADH and NADPH since they cannot be differentiated spectrally. The bulk of the evidence available would imply that the changes observed involve principally NADH, but this cannot be taken as conclusively established.

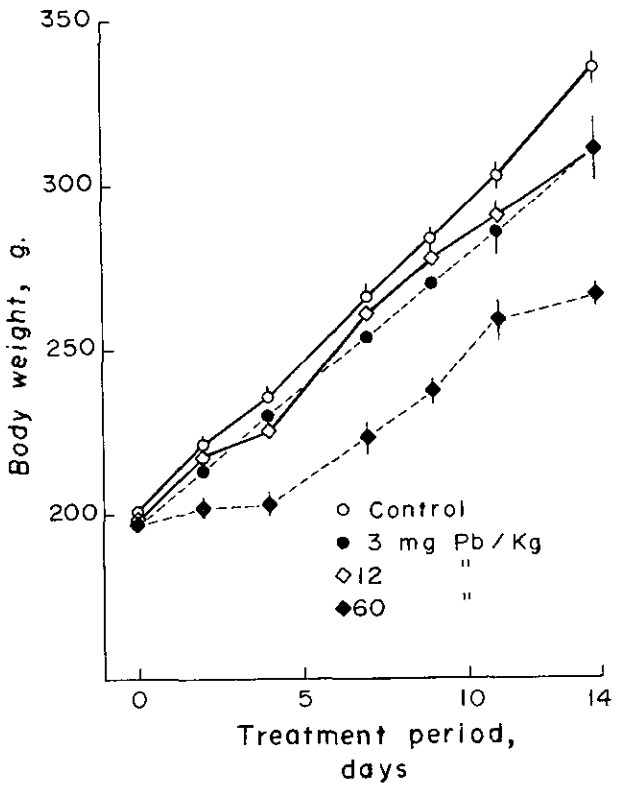

FIGURE 1. *Gross toxicity of the IP injection schedule used for* administration of Pb as measured by body weight gain. Total doses indicated were administered in six equally divided doses over the 2-week period. Each group consisted of six animals and average values are indicated ± SEM.

developed in the response shows a clear relationship with the dose of lead, although the net increase in NAD(P)H was not substantially different at total doses of 12 and 60 mg/kg body weight. There was no suggestion of the effect at 3.0 mg Pb/kg. The comparability of the tissues used in these measurements was judged by the initial absorbance of cytochrome c + c_1 (550–540 nm). This value has been previously established to represent more than 90% of the total cytochrome c in the tissue (*5*). The values thus obtained were: control, 0.0140 ± 0.0009 O.D. units (± SEM of ten tissues); 3 mg/kg, 0.0148 ± 0.0014 (six tissues); 12 mg/kg, 0.0146 ± 0.0013 (seven tissues); and 60 mg/kg, 0.0148 ± 0.0008 (seven tissues).

Figure 3 illustrates the change induced in the respiratory responses of cerebral cortex slices by treatment of animals with a total dose of 60 mg Pb/kg. In confirmation of the spectral measurements, an inhibition of potassium-induced respiration was observed. It was interesting to note that the inhibition showed some specificity for the

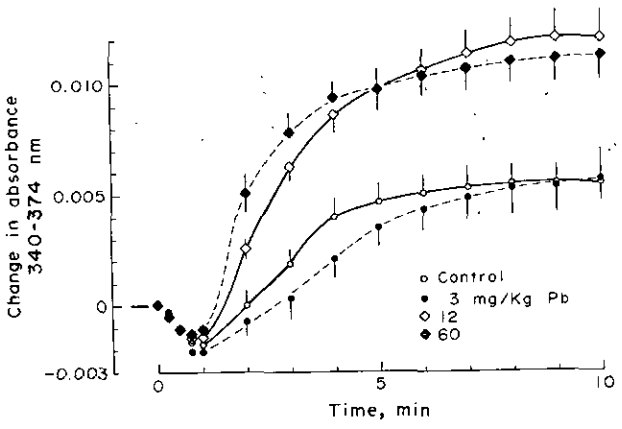

FIGURE 2. Effect of Pb on potassium-induced redox changes of NAD(P)H in cerebral cortex slices taken from exposed animals. HCO_3/CO_2 media was utilized with glucose as substrate. Same dosage schedule as in Fig. 1; 27 *mM* potassium added at zero time. Curves derived from the average of not less than six animals ± SEM.

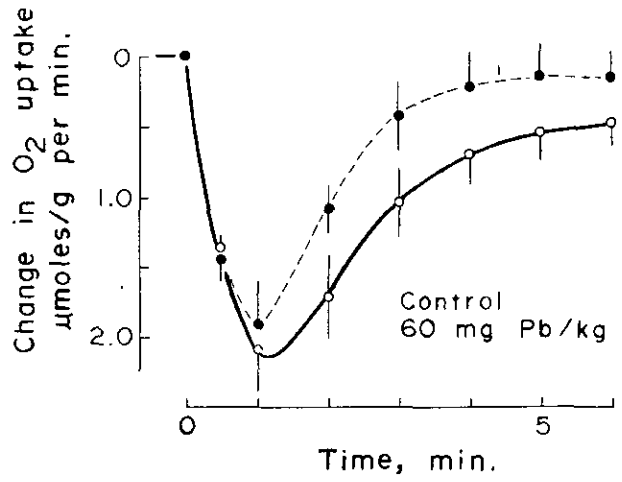

FIGURE 3. Potassium-induced respiratory changes in cerebral cortex slices taken from animals exposed to Pb. Same conditions as in Fig. 2. Each curve is an average derived from at least 15 animals and expressed ± SEM.

declining phase of the response, the peak rate showing little change from control tissues. The rates measured at 2 and 3 min following addition of potassium were statistically different from control ($p < 0.05$), whereas other values were nonsignificant ($p > 0.05$). Lead treatment appeared to have no effect upon the nonstimulated rate of respiration (1.63 ± 0.19 μmole/g min for control tissues and 1.60 ± 0.31 for tissues taken from lead-exposed animals).

Despite the apparent increased level of reduction of tissue pyridine nucleotides, neither

unstimulated or stimulated rates of aerobic glycolysis were significantly increased by the lead treatment (Fig. 4). In fact, a small statistically insignificant decrease in the stimulated lactic acid output by the tissues was observed following lead treatment.

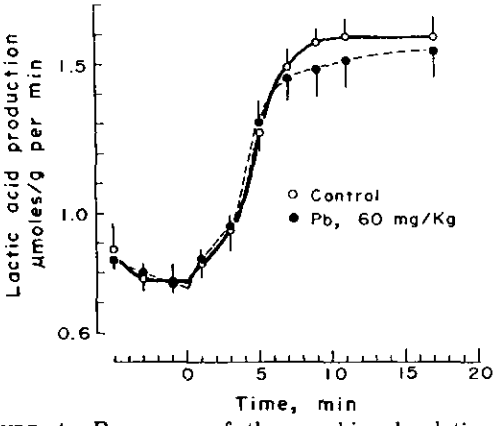

FIGURE 4. Responses of the aerobic glycolytic rate to potassium in cerebral cortex slices taken from Pb-exposed animals. Same conditions as in Fig. 2. Each curve represents the average of not less than 6 animals. Vertical bars indicate ± SEM.

The effects of lead on the metabolic responses of tissues taken from exposed animals could be largely reproduced *in vitro*. For these experiments its was necessary to change to a glycylglycine buffered incubation media, since lead salts were insoluble in the bicarbonate buffer ordinarily used. This resulted in substantial changes in the spectral responses and measured rates of aerobic glycolysis (compare Figs. 2, 4, and 5). These alterations are attributable primarily to the removal of HCO_3^-/CO_2 from the media rather than a toxic effect of glycylglycine (R. J. Bull, unpublished results).

Figure 5 shows the results obtained from tissues utilizing glucose as substrate when exposed to $67\mu M$ $PbCl_2$ 20 min prior to stimulation by potassium. Despite the fact that the reduction of NAD(P) was substantially reduced in the glycylglycine buffer, inclusion of lead in the incubation media resulted in an increased level of NAD(P)H following potassium relative to control. $PbCl_2$ at a concentration of $25\mu M$ was without effect. In contrast to the results obtained from tissues taken from exposed animals, a small but significant reduction in both nonstimulated and potassium-stimulated aerobic glycolysis was observed with lead, *in vitro*. The burst of respiration induced by the 3 to $30mM$ increase in media

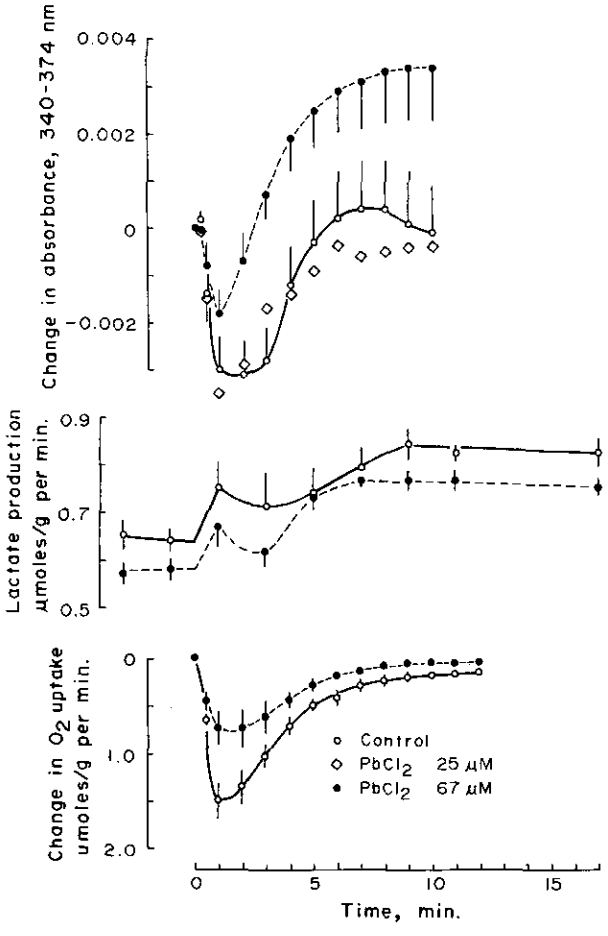

FIGURE 5. Effect on Pb, *in vitro*, on the metabolic responses of rat cerebral cortex slices to elecated potassium concentrations. Glucose as substrate, glycylglycine buffer: (top) NAD(P)H redox state; (center) aerobic glycolysis; (bottom) oxygen consumption. Each curve represents the average of not less than five tissues. Vertical bars indicate ± SEM.

potassium concentration was inhibited by approximately 50% by $67\ \mu M$ $PbCl_2$. Little specificity was noted for the declining phase of the response.

The effect of lead on the metabolic responses was essentially absent in cortical slices metabolizing pyruvate (Fig. 6). A small inhibition of the initial oxidation of NAD(P)H was noted, but there was no sign of the enhanced reduction of NAD(P) in the second phase of the response. Potassium-stimulated respiration of slices metabolizing pyruvate in the presence of $67\ \mu M$ $PbCl_2$ was not altered from control.

When lactate, $10mM$, was utilized as substrate the potassium-induced respiratory burst was inhibited by $67\ \mu M$ $PbCl_2$ (Fig. 7). The inhibition observed was approximately 30–35% during the peak

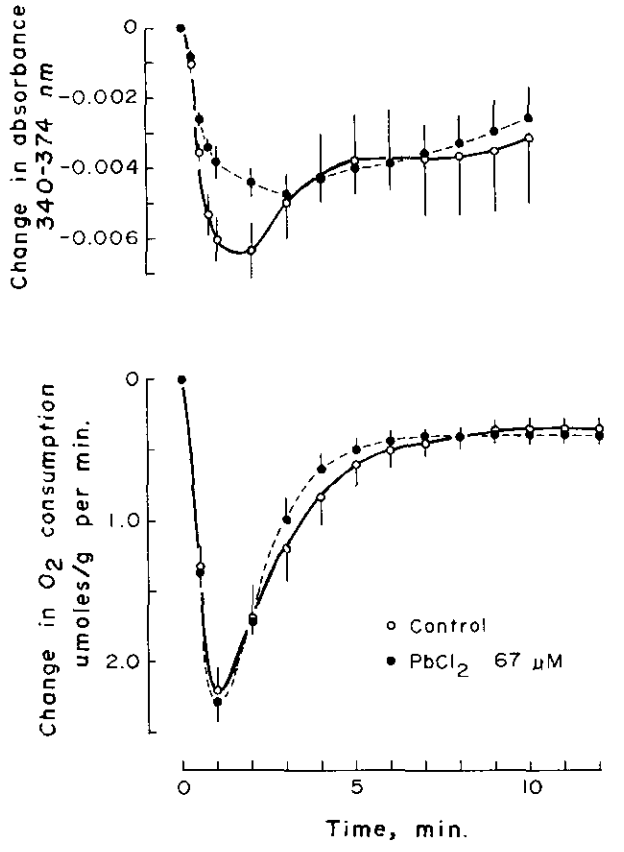

FIGURE 6. Effect of Pb, *in vitro*, on potassium-induced metabolic changes in rat cerebral cortex slices utilizing pyruvate as substrate. (top) NAD(P)H redox state; (bottom) oxygen consumption. Each curve represents the average of at least five tissues. Vertical bars indicate ± SEM.

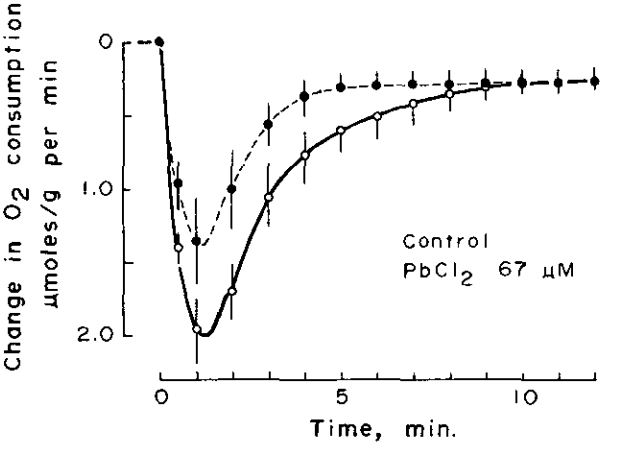

FIGURE 7. Effect of Pb, *in vitro*, on potassium-induced respiration in cerebral cortex slices utilizing lactate as substrate. Curves describe average results from at least 5 tissues. Vertical bars indicate ± SEM.

of the response. Again, little specificity was noted for any particular portion of the response.

The specificity of the lead effect for substrates donating a cytoplasmic reducing equivalent could also be demonstrated in tissues taken from lead-exposed animals. If pyruvate served as substrate, no significant alterations in the potassium-induced respiratory burst could be demonstrated as a total dose of 60 mg Pb/kg (Fig. 8).

Lead concentrations measured in whole blood and cerebral cortex of rats subjected to the IP injection schedule are shown in Table 1. Both the levels in blood and brain were found to increase in a manner directly dependent upon dosage. The concentrations of lead in blood were quite variable, particularly at the 60 mg/kg dose. If two samples of about 1000 μg/100 ml are excluded from this group, the remaining four animals averaged approximately 100 μg/100 ml. The great variability of

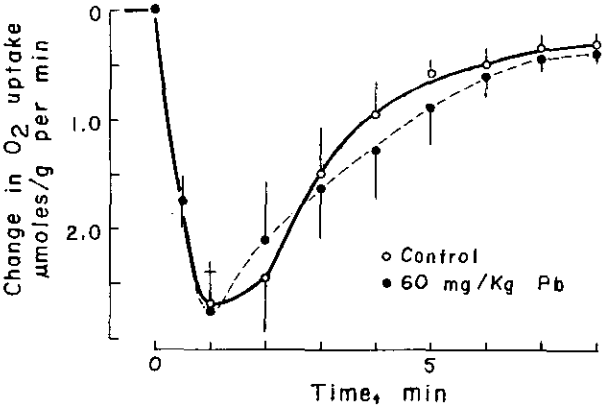

FIGURE 8. Respiratory responses of cerebral cortex slices, taken from animals exposed to Pb, to elevated potassium concentration when utilizing pyruvate as substrate. Same dosage schedule and conditions as utilized in Fig. 2. Each curve represents the average for at least six animals ± SEM.

Table 1. Lead concentrations in blood and brain following a 2-week, I.P. injection schedule.

	Pb in blood, μg/100 ml[a]		Pb in cerebral cortex, μg/g[a]	
Dose, mg/kg	Avg.	Range	Avg.	Range
Control	0.8	< 0.5 – 2.0	0.06	0.04 – 0.10
3.0	13.2	10.0 – 17.5	0.17	0.10 – 0.24
12.0	72.6	32.0 – 126	0.41	0.34 – 0.52
60	380	60 – 1040	1.02	0.48 – 1.78

[a] $n = 6$

blood concentrations of lead in this group was undoubtedly attributable to the tendency of lead, given in this dosage range, to precipitate in the peritoneal cavity, thus retarding systemic absorption. Cerebral cortical lead concentrations were also variable in this group, and an apparent relationship existed between unusually high blood level and higher concentrations in this tissue. However, too few samples were available for detailed analysis.

Discussion

To our knowledge, the present work represented the first attempt to document the effects of lead on energy metabolism of brain in a dynamic state. Cerebral cortex slices were induced to perform osmotic work in response to a change in media potassium concentration. The osmotic work cannot be exclusively attributed to transport of potassium, since calcium movements have also been implicated (*6*). Signs of an immediate energy demand were quickly followed by the reinstitution of metabolic control and the establishment of a new steady state characterized by recovery of ATP and phosphocreatine concentrations, slowing of the rate of respiration and reduction of the respiratory carriers (*4, 5*). It should be noted that rat brain has been estimated to respire at rates of 4.9 μmole O_2/g-min *in vivo* (*7*). In the isolated tissues utilized in the present study, such rates are approached only during the peak of the respiratory burst (1.6 basal plus 2.0 μmole/g-min during peak response). Therefore, the measured rates are completely reasonable in terms of the normal energy demand of the tissue, *in vivo*.

Previous work by Cremer (*8–11*) revealed a similar substrate selectivity for respiration supported by glucose in brain tissue of rats exposed to triethyltin and triethyllead. Both compounds inhibited $^{14}CO_2$ production from radiolabeled glucose but not from pyruvate in rat cortical slices. However, Cremer indicated that an increase in the rate of lactic acid production by brain slices accompanies the inhibition of glucose-supported respiration. This was not observed in the present work, although a substantial increase in tissue NAD(P)H was observed to occur following addition of potassium. Despite this difference, our data do strongly implicate an inhibition of the oxidation of reducing equivalents produced in the cytosol. This was the same explanation offered by Cremer for her results with organic lead and tin. Apparently, the increased NAD(P)H observed with inorganic lead is no longer simply associated with the cytoplasm, since it did not appear available for reduction of pyruvate to lactate. An alternate explanation is that pyruvate concentrations become limiting in our system as a result of increased Kreb's cycle oxidation and the huge sink for lactate produced by tissue superfusion. In either case, the increased NAD(P)H following addition of potassium must be derived from cytoplasmic oxidations in order to explain the differential effects of lead on glucose and pyruvate supported respiration within the framework of our present understanding of the intermediary metabolism of mammalian brain. That the inhibition of respiration observed was specifically associated with utilization of cytoplasmic reducing equivalents appears amply demonstrated by the results obtained with lactate serving as substrate. The only known pathway for lactate oxidation is through its conversion to pyruvate resulting in the production of 1 mole of NADH/mole of lactate oxidized.

In terms of the effects of lead on isolated mitochondria, there seems to be no easily discerned precedent for the specificity for the oxidation of cytosolic reducing equivalents. There seems to be general agreement that tissue pyridine nucleotides do not pass through the mitochondrial membrane and NADH reduced in the cytosol must be oxidized by shuttle mechanisms. Scott et al. (*12*) have reported the relative insensitivity of TMPD-ascorbate oxidation in isolated heart mitochondria but inhibition of succinate or NADH coupled respiration. However, in these same studies lead was observed to have various effects upon ion translocation in mitochondria depending upon the anionic composition of the media. In many respects, lead accumulation by mitochondria was found to be quite similar to the calcium accumulation reactions of isolated mitochondria. The present study utilized ionic stimulation to activate energy metabolism of brain slices and little alteration of metabolism was produced by lead in unstimulated tissues. This suggests that interactions of lead with ion transport mechanisms of brain mitochondria could underlie the effects noted in the present study. Indeed, calcium, a well-known antagonist of lead effects both *in vitro* and *in vivo*, has been shown (*13*) to influence the mitochondrial compartmentation of NADH.

We have reported somewhat similar results as noted here in brain slices taken from rats exposed to methylmercury (*14*). Coupled with the observations of Cremer (*8*) indicating similar findings with

triethyltin, triethyllead, and ethylmercury on brain, but not kidney slices, these data suggest a mechanism which may be common to heavy metal derivatives capable of penetrating the blood–brain barrier. Such an effect would explain the apparent selectivity certain of these compounds have for the central nervous system which is organized to preferentially utilize glucose as its major energy source both *in vivo* and *in vitro*.

On the basis of lead determinations in brain, the effect we observe seems relevant to current environmental exposures to lead. In an autopsy study involving 150 accident victims, Schroeder and Tipton (*15*) report an average of 0.1 μg Pb/g but a range of 80% of the samples which would encompass values to 1.4 μg/g. On a limited number of samples, Barry and Mossman (*16*) reported a similar average of 0.17 μg/g wet weight. However, Zaworski and Oyasu (*17*) reported brain levels averaging 0.5 μg/g in a survey of autopsy material from Chicago hospitals. The sample in this latter study was the right frontal lobe of the cerebral cortex. Levels averaging 0.41 μg Pb/g were observed to result in substantial alterations in the NAD(P)H response to elevated potassium concentrations in the present study. It is interesting to observe that Michaelson and Sauerhoff (*12*) reported concentrations of 0.5 μg/g in the brains of young rats displaying hyperactive behavior. To the extent that our data may be extrapolated to man, it would seem that relatively small elevations of brain lead concentrations over those encountered under current environmental conditions may produce biochemical changes in the brain.

The concentrations of lead required in the incubation media to produce similar effects *in vitro* were approximately 30-fold greater than those found in tissue taken from exposed animals. This suggests that a 20-min preincubation period *in vitro* was insufficient to allow lead to distribute in the tissue in a way similar to that occurring *in vivo*. This perhaps represents a limitation to *in vitro* experimentation with inorganic lead.

At this point it is not possible to project the types of functional impairment which would result from such changes at the biochemical level. From what is known of brain metabolism it would be supposed that the effects observed would be quite diverse and nonspecific in terms of the various functions of the nervous system. This indeed seems to be the picture observed with lead poisoning and has contributed to the difficulties encountered in attempting to understand the underlying mechanisms involved (*2*). What specificity is observed may depend largely upon the distribution of lead within the brain and not to any inherent susceptibility of particular cells of the brain. This is actually suggested by observations concerning the order in which histopathological damage develops as the result of high pre- and postnatal doses of lead (*1*).

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